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(54) Title: GENE THERAPY FOR INHIBITION OF ANGIOGENESIS (57) Abstract <p>The present invention relates to methods of gene therapy for inhibiting angiogenesis associated with solid tumor growth, tumor metastasis, inflammation, psoriasis, rheumatoid arthritis, hemangiomas, diabetic retinopathy, angiofibromas, and macular degeneration. Gene therapy methodology is disclosed for inhibition of primary tumor growth and metastasis by gene transfer of a nucleotide sequence encoding a soluble form of a VEGF tyrosine kinase receptor to a mammalian host. The transferred nucleotide sequence transcribes mRNA and a soluble receptor protein which binds to VEGF in extracellular regions adjacent to the primary tumor and vascular endothelial cells. Formation of a sVEGF-R/VEGF complex will prevent binding of VEGF to the KDR and FLT-1 tyrosine kinase receptors, antagonizing transduction of the normal intracellular signals associated with vascular endothelial cell-induced tumor angiogenesis. In addition, expression of a soluble receptor tyrosine kinase may also impart a therapeutic effect by binding either with or without VEGFs to form non-functional heterodimers with full-length VEGF-specific tyrosine kinase receptors and thereby inhibiting the mitogenic and angiogenic activities of VEGFs.</p>		

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TITLE OF THE INVENTION
GENE THERAPY FOR INHIBITION OF ANGIOGENESIS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part application of U.S. Provisional Application Serial No. 60/026,641, filed September 24, 1996.

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

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Not applicable.

FIELD OF THE INVENTION

20 The present invention relates to methods of gene therapy for inhibiting angiogenesis associated with tumor growth, inflammation, psoriasis, rheumatoid arthritis, hemangiomas, diabetic retinopathy, angiofibromas, and macular degeneration.

25 This invention also relates to animal models useful in the investigation of gene therapy-mediated inhibition of angiogenesis. The invention also relates to recombinant vectors which are useful in the disclosed gene therapy methods.

BACKGROUND OF THE INVENTION

Vascular endothelial cells form a luminal non-thrombogenic monolayer throughout the vascular system. Mitogens promote embryonic vascular development, growth, repair and angiogenesis in these cells. Angiogenesis involves the proteolytic degradation of the basement membrane on which endothelial cells reside followed by the subsequent chemotactic migration and mitosis of these cells to support sustained growth of a new capillary shoot. One class of mitogens selective for vascular endothelial cells include vascular endothelial growth factor (referred to as VEGF or VEGF-A) and the homologues placenta growth factor (PlGF), VEGF-B and VEGF-C.

Human VEGF exists as a glycosylated homodimer in one of five mature processed forms containing 206, 189, 165, 145 and 121 amino acids, the most prevalent being the 165 amino acid form.

U.S. Patent No. 5,240,848 discloses the nucleotide and amino acid sequence encoding the 189 amino acid form of human VEGF.

U.S. Patent No. 5,332,671 discloses the nucleotide and amino acid sequence encoding the 165 amino acid form of human VEGF.

Charnock-Jones et al (1993, *Biol. Reproduction* 48: 1120-1128) discloses the VEGF145 splice variant m RNA.

U.S. Patent No. 5,194,596 discloses the nucleotide and amino acid sequence encoding the 121 amino acid form of human VEGF.

The 206 amino acid and 189 amino acid forms of human VEGF each contain a highly basic 24-amino acid insert that promotes tight binding to heparin, and presumably, heparin proteoglycans on cellular surfaces and within extracellular matrices (Ferrara, et al., 1991, *J. Cell. Biochem.* 47: 211-218). The VEGF165 form binds heparin to a lesser extent while VEGF121 does not bind heparin.

Human PlGF is also a glycosylated homodimer which shares 46% homology with VEGF at the protein level. Differential

splicing of human PlGF mRNA leads to either a 170 amino acid or 149 amino acid precursor, which are proteolytically processed to mature forms of 152 or 131 amino acids in length, respectively (Maglione, et al., 1993, *Oncogene* 8: 925-931; Hauser and Weich, 1993, *Growth Factors* 9: 259-268).

VEGF-B was recently isolated and characterized (Olofsson, et al., 1996, *Proc. Natl. Acad. Sci.* 93: 2576-2581; Grimmond et al., 1996, *Genome Research* 6: 124-131). The full length human cDNAs encode 188 and 207 amino acid precursors wherein the NH₂ terminal portions are proteolytically processed to mature forms 167 and 186 amino acids in length. Human VEGF-B expression was found predominantly in heart and skeletal muscle as a disulfide-linked homodimer. However, human VEGF-B may also form a heterodimer with VEGF (*id.* @ 2580).

VEGF-C has also recently been isolated and characterized (Joukov, et al., 1996, *EMBO J.* 15: 290-298). A cDNA encoding VEGF-C was obtained from a human prostatic adenocarcinoma cell line. A 32 kDa precursor protein is proteolytically processed to generate the mature 23 kDa form, which binds the receptor tyrosine kinase, Flt-4.

VEGF-D was identified in an EST library, the full-length coding region was cloned and recognized to be most homologous to VEGF-C among the VEGF family amino acid sequences (Yamada, et al., 1997, *Genomics* 42:483-488). The human VEGF-D mRNA was shown to be expressed in lung and muscle.

VEGF and its homologues impart activity by binding to vascular endothelial cell plasma membrane-spanning tyrosine kinase receptors which then activates signal transduction and cellular signals. The Flt receptor family is a major tyrosine kinase receptor which binds VEGF with high affinity. At present the flt receptor family includes flt-1 (Shibuya, et al., 1990, *Oncogene* 5: 519-524), KDR/flk-1 (Terman, et al., 1991, *Oncogene* 6: 1677-1683; Terman, et al., 1992, *Biochem. Biophys. Res. Commun.* 187: 1579-1586), and flt-4 (Pajusola, et al., 1992, *Cancer Res.* 52: 5738-5743).

The involvement of VEGF in promoting tumor angiogenesis has spawned studies investigating possible antagonists of the process.

Both polyclonal (Kondo, et al., 1993, *Biochem. Biophys. Res. Commun.* 194: 1234-1241) and monoclonal (Kim, et al., 1992, *Growth Factors* 7: 53-64; Kim, et al., 1993, *Nature* 362: 841-844) antibodies raised against VEGF have been shown to suppress VEGF activity *in vivo*. Anti-VEGF antibody strategies to interdict angiogenesis and its attendant tumor are also addressed in Kim et al. (1993, *Nature* 362: 841-844) and Asano et al. (1995, *Cancer Research* 55: 5296-5301).

Kendall and Thomas (1993, *Proc. Natl. Acad. Sci.* 90: 10705-10709) isolated and characterized a cDNA encoding a secreted soluble form of flt-1 from cultured human umbilical vein endothelial cells (HUVEC). The recombinant version of this protein was purified by binding to immobilized heparin. Isolated soluble flt-1 was shown to inhibit VEGF activity *in vitro*. No suggestion regarding gene transfer protocols were disclosed.

Millauer et al. (1994, *Nature* 367: 576-579) disclose *in vivo* inhibition of tumor angiogenesis by expression of an artificially generated flk-1 mutant in which the intracellular kinase domain but not the membrane-spanning anchor was deleted. The authors do not forward any teaching or suggestion that a soluble form of a VEGF tyrosine kinase receptor would be useful in gene therapy applications.

Neovascularization of malignant tumors is an integral process contributing to solid tumor growth and neoplastic progression (Kondo et al., 1993, *Biochemical & Biophysical Research Communications* 194: 1234-1241; Carrau et al., 1995, *Invasion & Metastasis* 15: 197-202). In this context, several studies have demonstrated a positive correlation between neovascularization in malignant tumors and poor clinical outcomes (Volm et al., 1996, *Anticancer Research* 16: 213-217; Toi et al., 1994, *Japanese Journal of Cancer Research* 85: 1045-1049; Shpitzer et al., 1996, *Archives of Otolaryngology -- Head & Neck Surgery*; 122: 865-868; Staibano et al., 1996, *Human Pathology* 27: 695-700; Giatromanolaki et al., 1996, *J. of Pathology* 179: 80-88). While the angiogenic process has several mediators, it appears that vascular endothelial growth factor (VEGF) may be a critical growth factor with respect to initiating the cascade of

events stimulating new blood vessel formation in several tumor types (Toi et al., 1996, *Cancer* 77: 1101-1106; Maeda et al., 1996, *Cancer* 77: 858-63; Anan et al., 1996, *Surgery* 119: 333-339).

Aiello et al. (1995, *Proc. Natl. Acad. Sci. USA* 92:10457-10461) disclose genetically engineered chimeric extracellular VEGF receptors to block angiogenesis in non-malignant cells.

Despite recent advances in identifying genes encoding ligands and receptors involved in angiogenesis, no gene therapy application has been forwarded which overcomes the deleterious effect this process has in promoting primary tumor growth and subsequent metastasis. The present invention addresses and meets this need.

SUMMARY OF THE INVENTION

The present invention relates to methods of gene therapy for inhibiting VEGF-induced angiogenesis associated with diseases and disorders including, but not limited to, solid tumor growth, tumor metastasis, inflammation, psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas, diabetic retinopathy, and macular degeneration. These disorders are related in that VEGF acts as a mitogen to stimulate local angiogenesis from vascular endothelial cells which in turns exacerbates the condition.

The present invention relates to gene transfer of a DNA vector and concomitant *in vivo* expression of a soluble form of a tyrosine receptor kinase (sVEGF-R) within the mammalian host which binds VEGF or a VEGF homologue in and around the localized site of the disorder. The formation of a sVEGF-R/VEGF complex will inhibit binding of VEGF to the FLT-1 and KDR tyrosine kinase receptors spanning the vascular endothelial cell membrane, thus preventing initiation of the signal transduction stimulating angiogenesis. In addition, expression of sVEGF-R may also impart a therapeutic effect by binding to membrane associated VEGF-Rs. VEGF-Rs are thought to be dimerized by binding dimeric VEGF ligand which in turn allows the receptor intracellular tyrosine kinase domains to transphosphorylate each other generating phosphorylated tyrosine residues that facilitate

the subsequent binding and activation of downstream signal transduction proteins. sVEGF-Rs can form heterodimers with full-length VEGF-Rs that, because the sVEGF-Rs are devoid of an intracellular tyrosine kinase region, prevent receptor tyrosine kinase domain transphosphorylation, the initiation of signal transduction and thus VEGF-induced mitogenesis and angiogenesis in a dominant negative manner.

A nucleotide sequence encoding a sVEGF-R for inclusion in a gene therapy vector of the present invention may be chosen from a group of genes encoding tyrosine kinase receptors, namely from the group consisting of *sflt-1*, *flt-1*, KDR (also denoted *flk-1*), and *flt-4*. The resulting DNA fragment encodes a protein or protein fragment which binds VEGF and/or KDR/*flk-1* and inhibits formation of a wild-type, functional VEGF-R/VEGF complex.

A preferred application of the present invention relates to promoting inhibition of solid tumor angiogenesis and metastasis by utilizing the disclosed gene therapy methodology. In particular, methods are disclosed for inhibition of primary tumor growth and metastasis by gene transfer of a nucleotide sequence encoding sVEGF-R to a mammalian host. The transferred nucleotide sequence transcribes mRNA and expresses sVEGF-R such that sVEGF-R binds to VEGF in extracellular regions adjacent to the primary tumor and vascular endothelial cells. Formation of a sVEGF-R/VEGF complex will prevent binding of VEGF to the KDR and FLT-1 tyrosine kinase receptors, antagonizing transduction of the normal intracellular signals associated with vascular endothelial cell-induced tumor angiogenesis. In addition, expression of sFLT-1 may also impart a therapeutic effect by binding either with or without VEGFs to form non-functional heterodimers with full-length VEGF-Rs and thereby inhibiting the mitogenic and angiogenic activities of VEGFs.

In a particular embodiment of the present invention a truncated version of a soluble or transmembrane form of FLT-1 (Shibuya, et al., 1990, *Oncogene* 5: 519-524) is utilized in gene therapy protocols. It will be within the purview of the skilled artisan to generate

a sVEGF-R or VEGF-RTMI construct expressing a truncated FLT-1 protein which binds to VEGF, a VEGF homologue and/or dimerizes with a full-length VEGF-R inhibiting its activation on the surface plasma membrane of vascular endothelial cells (Figure 1). Such a
5 construct may be generated by recombinant DNA techniques known in the art using a DNA fragment encoding a partial or complete amino acid sequence of a FLT receptor. Using recombinant DNA techniques, DNA molecules are constructed which encode at least a portion of the VEGF receptor capable of binding VEGF without stimulating either
10 mitogenesis or angiogenesis. Standard recombinant DNA techniques are used such as those found in Maniatis, et al. (1982, Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

In another embodiment of the present invention a mutated
15 version of FLT-1 is generated which is defective in protein kinase activity, namely a FLT-1 protein mutated at or around one or more known active sites for protein kinase activity. A *flt-1* construction will express the extracellular domain, transmembrane domain and the mutated portion of the intracellular domain such that the resulting
20 protein at least substantially inhibits related intracellular protein kinase activity.

In a preferred embodiment of the present invention, a naturally expressed alternatively spliced DNA encoding a soluble form of FLT-1 (Kendall and Thomas, 1993, *Proc. Natl. Acad. Sci.* 90: 10705-
25 10709; U.S. Application Serial No. 08/232,538, hereby incorporated by reference; described herein as sVEGF-RI or sFLT-1 and listed as SEQ ID NO:1 (nucleotide sequence) and SEQ NO ID:2 (amino acid sequence) is the template for constructing a gene therapy vector wherein either
30 expressed sFLT-1 or a biologically active truncated form binds VEGF and inhibits complex formation, dimerization and activation of full-length VEGF-Rs, and hence, pathological angiogenesis.

The present invention relates to both viral and non-viral recombinant vectors for delivery to the target hosts. To this end, a preferred non-viral recombinant plasmid described herein is

pcDNA3/sflt-1. An especially preferred recombinant plasmid of the present invention is pcDNA1AsFLT-1, as described in Example Section 5.

A recombinant adenovirus (Ad) system is preferred for delivery and prolonged expression within target cells proximal to a solid tumor. A particularly useful adenovirus system used in the present invention is described in Example 4.

Any sVEGF-R construct, including but in no way limited to sVEGF-RI and biologically active truncated forms, may be delivered to the mammalian host using a vector or other delivery vehicle. DNA delivery vehicles can include viral vectors such as adenoviruses, adeno-associated viruses, and retroviral vectors. See, for example: Chu et al., 1994, *Gene Therapy* 1: 292-299; Couture et al., 1994, *Hum. Gene Therapy* 5:, 667-277; and Eiverhand et al., 1995, *Gene Therapy* 2:336-343. Non-viral vectors which are also suitable include naked DNA (see Example Sections 1, 2, 3, and 5), DNA-lipid complexes, for example liposome-mediated or ligand/ poly-L-Lysine conjugates, such as asialoglycoprotein-mediated delivery systems. See for example: Felgner et al., 1994, *J. Biol. Chem.* 269:2550-2561; Derossi et al., 1995, *Restor. Neurol. Neuros.* 8:7-10; and Abcallah et al., 1995, *Biol. Cell* 85:1-7. It is preferred that local cells such as adipose tissue cells or smooth muscle cells, as well as tumor cells, be targeted for delivery and concomitant *in vivo* expression of the respective sVEGF-R protein to promote inhibition of tumor angiogenesis.

A recombinant Ad/sVEGF-RI is a preferred virus for targeting cells proximal to a solid tumor.

An especially preferred recombinant Ad/sVEGF-RI virus is AdHCMV_sFLT-1.

Another especially preferred recombinant Ad/sVEGF-RI virus is AdHCMVI_{1s}FLT .

Any membrane bound (mVEGF-R) construct or any FLT-1 or KDR construct encoding a protein deficient in kinase activity may be targeted primarily to vascular endothelial cells in the vicinity of tumor growth. DNA delivery vehicles described above may be utilized to target

any such gene transfer construct to vascular endothelial cells of the mammalian host.

As used herein, "VEGF" or "VEFG-A" refers to vascular endothelial growth factor.

5 As used herein, "homologue of VEGF" refers to homodimers of VEGF-B, VEGF-C, VEGF-D and PlGF and any functional heterodimers formed between VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF, including but not limited to a VEGF-A/PlGF heterodimer.

10 As used herein, "VEGF-B" refers to vascular endothelial growth factor-B.

As used herein, "VEGF-C" refers to vascular endothelial growth factor-C.

15 As used herein, "VEGF-D" refers to vascular endothelial growth factor-D.

As used herein, "KDR" or "FLK-1" refers to kinase insert domain-containing receptor or fetal liver kinase.

As used herein, "FLT-1" refers to fms-like tyrosine kinase receptor.

20 As used herein, "Ad" refers to adenovirus.

As used herein, "HUVEC" refers to human umbilical vein endothelial cell(s).

As used herein, the term "mammalian host" refers to any mammal, including a human being.

25 As used herein, "sVEGF-R" generically refers to a soluble form of a tyrosine kinase receptor which binds to its respective vascular endothelial growth factor such as VEGF, VEGF-B, VEGF-C, VEGF-D and PlGF without stimulating receptor activation, mitogenesis of vascular endothelial cells or angiogenesis.

30 As used herein, "sVEGF-RI" or "sFLT-1" refers to the native human soluble form of sFLT, disclosed in U.S. Application Serial No.08/232,538 and presented herein in cDNA form (comprising SEQ ID NO:1) and protein form (SEQ ID NO:2).

As used herein, "VEGF-Rs" refers to a human wild-type VEGF/VEGF homologue specific tyrosine kinase receptor such as FLT-1 and KDR.

5 As used herein, "mVEGF-R" generically refers to a human wild-type VEGF/VEGF homologue specific tyrosine kinase receptor such which is membrane bound , including but not limited to FLT-1, VEGF-RTMI, KDR, and VEGF-RTMII, as shown in Figure 1.

It is an object of the present invention to provide gene therapy methods to inhibit angiogenesis and growth of solid tumors.

10 It is also an object of the present invention to utilize a gene or gene fragment of sVEGF-R in gene therapy methods to inhibit angiogenesis and growth of solid tumors.

It is also an object of the present invention to utilize sVEGF-RI in gene therapy methods to inhibit angiogenesis and growth of solid tumors.

15 It is an object of the present invention to disclose animal models for the determination of efficacy of FLT-1-based constructions for cell delivery and *in vivo* expression in the mammalian host.

It is an object of the present invention to provide recombinant DNA vectors containing sVEGF-RI constructs for use in gene therapy to locally inhibit angiogenesis in a mammalian host.

BRIEF DESCRIPTION OF THE FIGURES

25 Figure 1 shows a schematic diagram of full length VEGF-Rs (FLT-1 and KDR), the soluble VEGF receptors (sVEGF-RI and sVEGF-RII) and the soluble receptors containing the C-terminal transmembrane region (sVEGF-RTMI and sVEGF-RTMII), with the protein domains of each.

Figure 2 shows the nucleotide sequence of which encodes human sFLT-1 [sVEGF-RI] (SEQ ID NO:1).

30 Figure 3A and Figure 3B show the amino acid sequence of human sFLT-1 [sVEGF-RI] (SEQ ID NO:2).

Figure 4 shows inhibition of tumor nodules grown in nude mice for HT-1080 mouse cells transiently transfected with pcDNA3/*sflt-1* (○) or pcDNA3 (●). 3×10^6 cells were injected at day 0.

5 Figure 5 shows inhibition of tumor nodules grown in nude mice for HT-1080 mouse cells stably transfected with pcDNA3/*sflt-1* (●) or pcDNA3 (○).

Figure 6 shows the survival plot of scid mice injected with (a) D-54MG human glioblastoma cells stably transfected with pcDNA 3 (■); (b) D-54MG human glioblastoma cells stably transfected with
10 pcDNA-*sflt-1* (□); and (c) untransfected D-54MG human glioblastoma cells (▣).

Figure 7 shows additional data points from the experiment detailed in Figure 6, namely that a CB-17 scid-mouse human-glioma model was used to assess the effect of stable *sflt-1* expression on tumor
15 growth and survival. (■)D-54MG human glioblastoma cells stably transfected with pcDNA3; (b) D-54MG human glioblastoma cells stably transfected with pcDNA-*sflt-1* (▲); and (c) untransfected D-54MG human glioblastoma cells (○).

Figure 8 shows that tumor growth in mice as measured by
20 average volume and mass (\pm SD) was significantly inhibited by expression of the *sflt-1* gene (1-tailed Student's t-test, $p < 0.0001$ for comparison of masses) subcloned into pCDNA1A3, resulting in pcDNAIA3FLT-1.

Figure 9 shows that the tumor masses in mice of *sFlt-1*
25 expressing cells were significantly smaller than either the adenovirus treated control cells ($p = 0.035$) or the no virus treated control cells ($p = 0.007$) using the appropriate 1-tailed Student's t-test.

30 DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of gene therapy for inhibiting VEGF-induced angiogenesis associated with diseases and disorders including, but not limited to, solid tumor growth, tumor metastasis, inflammation, psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas, diabetic retinopathy, and macular

degeneration. These disorders are related in that VEGF acts as a vascular endothelial cell mitogen and chemotactic agent to stimulate local angiogenesis which in turns exacerbates the condition.

The present invention relates to gene transfer of a DNA
5 vector and concomitant *in vivo* expression of a soluble form of a VEGF receptor (sVEGF-R) within the mammalian host which binds VEGF or a VEGF homologue in and around the localized site of the disorder. The formation of a sVEGF-R/VEGF complex will inhibit binding of VEGF to the full-length KDR and FLT-1 tyrosine kinase receptors spanning the
10 vascular endothelial cell surface plasma membrane, thus preventing transduction of the mitogenic and other signals stimulating angiogenesis. In addition, expression of sVEGF-R may also impart a therapeutic effect by binding with membrane associated VEGF full-length receptors to form non-functional receptor heterodimers and
15 thereby inhibit the mitogenic activity of VEGF in a dominant negative manner.

A nucleotide sequence encoding a sVEGF-R for inclusion in a gene therapy vector of the present invention may be chosen from a group of genes encoding tyrosine kinase receptors, namely from the
20 group consisting of *sflt-1*, *flt-1*, KDR (also denoted *flk-1*), and *flt-4*. The resulting DNA fragment encodes a protein or protein fragment which binds VEGF and inhibits formation of a wild-type, functional VEGF-R/VEGF complex.

A preferred application of the present invention relates to
25 methods inhibiting solid tumor angiogenesis, tumor growth and metastasis by utilizing the disclosed gene therapy methodology. In particular, methods are disclosed for inhibition of primary tumor growth and metastasis by gene transfer of a nucleotide sequence encoding sVEGF-R to a mammalian host. The transferred nucleotide
30 sequence transcribes mRNA and expresses sVEGF-R such that sVEGF-R binds to VEGF in extracellular regions adjacent to the primary tumor and vascular endothelial cells and/or heterodimerizes with full-length VEGF-Rs inhibiting their function. Formation of a sVEGF-R/VEGF-R heterodimeric complexes will prevent VEGF-induced dimerization of

functional full-length VEGF-Rs, antagonizing receptor transphosphorylation-dependent signal transduction associated with vascular endothelial cell-activation and tumor angiogenesis. In addition, expression of sVEGF-R may also impart a therapeutic effect by binding either with or without VEGFs to form non-functional heterodimers with full-length VEGF-Rs and thereby inhibiting the mitogenic and angiogenic activities of VEGFs in constructing the necessary DNA vector. Restriction endonuclease cleavage sites are identified within the receptor DNA and can be utilized directly to excise the extracellular-encoding portion. In addition, PCR techniques as described above may be utilized to produce the desired portion of DNA. It is readily apparent to those skilled in the art that other techniques, which are standard in the art, may be utilized to produce sVEGF-R molecules in a manner analogous to those described above. Such techniques are found, for example, in Maniatis et al. (1982, Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

In a particular embodiment of the present invention a DNA fragment encoding a soluble form of the FLT-1 amino acid sequence (see Shibuya, et al., 1990, *Oncogene* 5: 519-524) is utilized in gene therapy protocols. It will be within the purview of the skilled artisan to generate a sVEGF-R construct which binds to VEGF and inhibits forming a complex with wild-type full-length VEGF-R dimers on the cell surface membrane of vascular endothelial cells. Such a construct may be generated by recombinant DNA techniques known in the art using a DNA fragment encoding a partial or complete amino acid sequence of a FLT receptor. Using recombinant DNA techniques, DNA molecules are constructed which encode at least a portion of the VEGF receptor capable of binding VEGF without stimulating mitogenesis or angiogenesis. As described below, *in vivo* delivery of a DNA construct encoding sVEGF-R is targeted to cells and tissue which surround the tumor, including but not limited to vascular endothelial cells, muscle cells, adipose cells, as well as tumor cells and surrounding tissues such as muscle tissue and adipose tissue.

The present invention also relates to therapeutic treatment of the metastatic spread of tumors, the principal cause cancer mortality. Tumor cells can metastasize by entry into the circulatory system, transport to distant sites, implantation back into the surrounding tissue and growth. Inhibition of any step in this process would be expected to inhibit the ultimate establishment and growth of metastatic foci. To this end, an additional aspect of the present invention relates to use of the gene therapy constructs of the present invention, including but not limited to sFlt, to inhibit the metastatic spread of tumors. The significant inhibition of the establishment of HT1080 metastatic lung foci by sflt expression as shown in Example 2 shows that sflt is effective in inhibiting this process. The sflt-1-transfected HT1080 cell tail vein injection experiment monitors implantation and/or growth of circulating tumor cells, two of the crucial steps in metastatic spread. It is envisioned that sflt may decrease the efficiency of tumor cell extravasation out of blood and into surrounding tissue, possibly by inhibiting VEGF-induced vascular permeability which could facilitate cell migration through vessel walls. Additionally, expression of sFlt is expected to arrest neovascular development within metastatic foci thus diminishing their growth and/or viability.

In another particular embodiment of the present invention a DNA fragment encoding the extracellular ligand binding domain and the transmembrane domain of FLT-1 (see Figure 1) is utilized in gene therapy protocols. Such a DNA construct may be constructed to contain the appropriate wild-type signal sequence such that the proper insertion into the plasma membrane occurs. To this end, it is preferred that viral and non-viral constructs which express VEGF-RTMI (Figure 1) or a biological equivalent thereof, will be targeted substantially to vascular endothelial cells within the region of the tumor.

In another specific embodiment of the present invention, flt-1 is utilized as a template to generate a mutated version of FLT-1 defective in protein kinase activity. A mutant in this class would possess one or more mutations at or around one or more known active sites for protein kinase activity. In other words, the mutant FLT-1 protein will

comprise an extracellular domain, a transmembrane domain, and a mutated intracellular domain. As noted in the previous paragraph regarding delivery of VEGF-RTMI, it is preferred that viral and non-viral constructs which express a mutant FLT-1 be targeted substantially to vascular endothelial cells within the region of the tumor.

An especially preferred template for practicing the present invention is the cDNA encoding a soluble form of FLT-1 (sVEGF-RI), described in Kendall and Thomas (1993, *Proc. Natl. Acad. Sci.* 90: 10705-10709) and U.S. Application Serial No. 08/232,538 which is hereby incorporated by reference. Briefly, a cDNA clone encoding sVEGF-RI was isolated in a two-stage approach employing polymerase chain reaction (PCR) based technology and cDNA library screening. In the first stage, DNA oligonucleotides derived from the extracellular domain sequence information from the known full length FLT, KDR or other VEGF receptor is used to design oligonucleotide primers for the amplification of sVEGF-R-specific DNA fragments. In the second stage, these fragments are cloned to serve as probes for the isolation of complete sVEGF-R cDNA from a commercially available lambda gt10 cDNA library (Clontech) derived from HUVECs (ATCC CRL 1730). This sVEGF-RI cDNA expresses an alternatively spliced form of the FLT-1 precursor mRNA that includes 31 unique amino acid residues at the C-terminal end not found in FLT-1 (see Figure 2 and SEQ ID NO:1 for nucleotide sequence and Figure 3 and SEQ ID NO:2 for amino acid sequence). These 31 unique residues are encoded by an intron that is not removed in this alternatively spliced version. The alternatively spliced mRNA is translated into this intron region until the first stop codon is encountered. This especially preferred template (sflt-1 or sVEGF-RI) for a gene therapy vector will express sVEGF-RI *in vivo* and bind VEGF and/or heterodimerizes with full-length VEGF-Rs (e.g. VEGF-RI/FLT-1 and VEGF-RII/KDR), thus inhibiting tumor angiogenesis.

The cloned sVEGF-RI cDNA obtained through the methods described above may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into

prokaryotic or eukaryotic host cells to produce recombinant sVEGF-RI. Techniques for such manipulations are fully described in Maniatis, et al.(id.), and are well known in the art.

As noted above, a preferred embodiment of the present invention relates to methods of inhibiting angiogenesis of solid tumors to prevent further tumor growth and eventual metastasis. To this end, any solid tumor or the region surrounding the tumor accessible to gene transfer will be a target for the disclosed therapeutic applications. A sVEGF-R gene or gene fragment, including but not limited to sVEGF-RI and any biologically active truncated version, housed within a recombinant viral- or non-viral-based gene transfer system may be directed to target cells within proximity of the tumor by any number of procedures known in the art, including but not limited to (a) surgical procedures coupled with administration of an effective amount of the DNA to the site in and around the tumor (involving initial removal of a portion or the entire tumor, if possible); (b) injection of the gene transfer vehicle directly into or adjacent to the site of the tumor; and, (c) localized or systemic delivery of the gene transfer vector and/or gene product using techniques known in the art; as listed below.

Therefore, any solid tumor which contains VEGF expressing cells will be a potential target for treatment. Examples, but by no means listed as a limitation, of solid tumors which will be particularly vulnerable to sVEGF-R gene therapy applications are (a) neoplasms of the central nervous system such as, but again not necessarily limited to glioblastomas, astrocytomas, neuroblastomas, meningiomas, ependymomas; (b) cancers of hormone-dependent tissues such as prostate, testicals, uterus, cervix, ovary, mammary carcinomas including but not limited to carcinoma in situ, medullary carcinoma, tubular carcinoma, invasive (infiltrating) carcinomas and mucinous carcinomas; (c) melanomas, including but not limited to cutaneous and ocular melanomas; (d) cancers of the lung which at least include squamous cell carcinoma, spindle carcinoma, small cell carcinoma, adenocarcinoma and large cell carcinoma; and (e) cancers of the gastrointestinal system such as esophageal, stomach, small

intestine, colon, colorectal, rectal and anal region which at least include adenocarcinomas of the large bowel.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, fungal cells, yeast cells, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal or bacteria-insect cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant sVEGF-R in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant sVEGF-R expression, include but are not limited to,

Commercially available mammalian expression vectors which may be suitable for recombinant sVEGF-R expression, include but are not limited to, pcDNA3.1 (Invitrogen), pBlueBacHis2 (Invitrogen), pLITMUS28, pLITMUS29, pLITMUS38 and pLITMUS39 (New England Biolabs), pcDNA1, pcDNA1amp (Invitrogen), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pDBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and λ ZD35 (ATCC 37565).

DNA encoding a sVEGF-R, sVEGF-RI or truncated version thereof may also be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila, moth, mosquito and armyworm derived cell lines. The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, Ad/polylysine DNA complexes, protoplast fusion, and electroporation. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171) and HEK 293 cells. Insect cell lines which may be suitable and are commercially available include but are not limited to 3M-S (ATCC CRL 8851) moth (ATCC CCL 80) mosquito (ATCC CCL 194 and 195; ATCC CRL 1660 and 1591) and armyworm (Sf9, ATCC CRL 1711).

A DNA fragment encoding a sVEGF-R, sVEGF-RI or mutant versions thereof may be delivered either systemically or to target cells in the proximity of a solid tumor of the mammalian host by viral or non-viral based methods. Viral vector systems which may be utilized in the present invention include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors; (c) adeno-associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picarnovirus vectors; and (i) vaccinia virus vectors. Non-viral methods of delivery include but are not necessarily limited to direct injection of naked DNA, such as a recombinant DNA plasmid expression vector described herein comprising a DNA fragment encoding sVEGF-R, VEGF-RTM, or mutated forms of FLT-1 or KDR.

The present invention therefore relates to non-viral recombinant vectors for delivery to the target hosts. To this end, a preferred recombinant plasmid described herein is pcDNA3/sflt-1. An especially preferred recombinant plasmid of the present invention is pcDNAIAAsFLT-1, as described in Example Section 5.

A recombinant adenovirus (Ad) system is preferred for delivery and prolonged expression within target cells proximal to a solid tumor. A particularly useful adenovirus system used in the present invention is described in Example 4.

A recombinant Ad/sVEGF-RI is a preferred virus for targeting cells proximal to a solid tumor.

An especially preferred recombinant Ad/sVEGF-RI virus is AdHCMVsFLT-1.

Another especially preferred recombinant Ad/sVEGF-RI virus is AdHCMVI1sFLT .

The recombinant Ad/sVEGF-RI viruses of the present invention, including AdHCMVsFLT-1 and AdHCMVI1sFLT, are preferably administered to the host by direct injection into a solid tumor and/or quiescent tissue proximal to the solid tumor, such as adipose or muscle tissue. It will of course be useful to transfect tumor cells in the region of targeted adipose and muscle tissue. Transient expression of a sVEGF-R or VEGF-RTM in these surrounding cells will result in a local extracellular increase in these proteins and will promote binding with VEGF and full-length VEGF-Rs, thus inhibiting formation of activated full-length VEGF-R dimers.

The recombinant Ad/VEGF-RI viruses of the present invention, including AdHCMVsFLT-1 and AdHCMVI1sFLT, may also be delivered by i.v. injection. A recombinant adenovirus delivered by i.v. injection will preferentially infect hepatocytes when administered intravenously, where expression persists for approximately 3-4 weeks subsequent to the initial infection. Suitable titers will depend on a number of factors, such as the particular vector chosen, the host, strength of promoter used and the severity of the disease being treated.

The skilled artisan may alter the titer of virus administered to the patient, depending upon the method of delivery, size of the tumor and efficiency of expression from the recombinant virus. A dose in the range of 10^9 - 10^{11} pfu adenovirus is preferred to treat most primary tumors. The skilled artisan will also realize that the number of viral particles encoding the transgene, whether or not replication competent in a complementing host cell, are a relevant dosing unit. In most Adenovirus constructs, there are 50 to 100-fold more DNA containing particles than pfus.

Non-viral vectors which are also suitable include DNA-lipid complexes, for example liposome-mediated or ligand/ poly-L-Lysine conjugates, such as asialoglyco-protein-mediated delivery systems (see, e.g., Felgner et al., 1994, *J. Biol. Chem.* 269: 2550-2561; Derossi et al., 1995, *Restor. Neurol. Neuros.* 8: 7-10; and Abcallah et al., 1995, *Biol. Cell* 85: 1-7).

There are many embodiments of the instant invention which those skilled in the art can appreciate from the specification. To this end, different transcriptional promoters, terminators, carrier vectors or specific gene sequences may be used successfully.

The present invention provides methods of gene therapy which inhibit tumor angiogenesis in a mammalian host. It will be readily apparent to the skilled artisan that various forms of the nucleotide sequence(s) encoding FLT-1, sVEGF-RTMI, sVEGF-R, sVEGF-RI or any mutated version thereof may be utilized to alter the amino acid sequence of the expressed protein. The altered expressed protein may have an altered amino acid sequence, yet still bind to VEGF and in turn inhibit the molecular cascade required to stimulate tumor angiogenesis. For example, various COOH terminal truncated forms of sVEGF-RI are envisioned in the present invention. It will be of ease for the skilled artisan to generate such altered forms upon review of this specification. Any such truncated version of FLT which is soluble and which binds VEGF, a VEGF homologue and/or FLT-1 or KDR is considered a functional equivalent in light of the teachings of this specification. It is also envisioned, as described in the specification, that

membrane bound mutant forms, such as COOH-terminal deletion mutants of FLT-1 and point mutations in the intracellular kinase domain, resulting in a mutant protein substantially defective in protein kinase activity, may be useful as a gene therapy construct for patient delivery and in vivo expression so as to inhibit tumor angiogenesis.

The following examples are provided to illustrate the present invention without, however, limiting the same hereto.

EXAMPLE 1

Isolation of a cDNA Encoding Human sFLT-1

PCR derived products were used as hybridization probes for screening a lambda gt10 cDNA library derived from HUVECs (Clontech). Plating and plaque lifts of the library were performed by standard methods (Maniatis, et al., 1982, Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The probes were random-primed labeled with ^{32}P -dCTP to high specific activity and a separate screening of the library (1×10^6 plaques per screen) was conducted with each probe. The probes were added to hybridization buffer (50% formamide, 5 x Denhardtts, 6 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M $\text{Na}_3\text{citrate} \cdot 2\text{H}_2\text{O}$, pH 7.0), 0.1% SDS, 100 mg/ml salmon sperm DNA) at 1×10^6 cpm/ml.

Four positively hybridizing phage were detected using the *flt-1*-specific probe. These positively hybridizing phage were observed to be less than full length *flt-1*.

Two *flt-1* cDNA clones of about 2.0 kb and 2.7 kb in length were subcloned into pGEM vectors (Promega) and bi-directionally sequenced in their entirety by the chain termination method (Sanger et al., 1977, *Proc. Natl. Acad. Sci* 74: 5463-5467) and shown to contain a single open reading frame of about 569 amino acids. Sequence analysis demonstrated that a portion of the 5' *flt-1* coding region was missing from these clones. The remainder of the 5' end was cloned using PCR and combined with the DNA of the clones lacking the 5' end to yield a single open reading frame encoding about 687 amino acids.

The *flt-1*-derived sVEGF-RI (sflt-1) cDNA nucleotide sequence and deduced amino acid sequence is shown in Figure 2 (nucleotide sequence: SEQ ID NO: 1) and Figure 3 (amino acid sequence: SEQ ID NO: 2). Inspection of the deduced amino acid sequence reveals the presence of a single, large open reading frame of 687 amino acids. By comparison with amino acid sequence of the full length FLT-1 VEGF receptor, 31 amino acids are encoded at the C-terminal end of the sVEGF-RI cDNA which are different from those of FLT-1.

EXAMPLE 2

Inhibition of Tumor Angiogenesis in Mice by Administration of Cells Which Transiently Express sVEGF-RI

The sVEGF-RI cDNA described in Example 1, cloned in pGEM3z and referred to as psflt-1, was digested with BamHI, purified and ligated into BamHI-digested pcDNA3. The resulting plasmid, pcDNA3/sflt-1 (alternatively referred to as SFLT-1), was verified by restriction mapping as well as DNA sequencing of the 5' and 3' 500bp of the BamHI insert. The plasmid was transformed into Top10F' E. coli and purified using Qiagen mega prep and Qiagen Endotoxin removal kit.

The expression plasmid pcDNA3/sflt-1, was mixed with adenovirus-polylysine (AdPI) and transfected into mouse HT-1080 cells (ATCC CRL 1730). Control transfections were performed in identical fashion using unmodified pcDNA3. HT-1080 cells were transfected when 80% confluent and harvested 16-24 hours for subsequent study.

Cell counting on triplicate wells was performed for 3 time points within 7 days of transfection using trypan blue exclusion and revealed no difference in the growth curves between the two groups.

The harvested cells were injected either subcutaneously or via tail vein in nude mice and nodules were measured on selected days for the subcutaneous nodules. For the tail vein injections two sets of experiments were performed. In the first experiment, the animals were sacrificed prior to the development of nodules but there were detectable

differences in the weights of the lungs but the weights were not significantly different. In the second experiment there were definitive differences in the number of nodules per sections with the sflt-1 group having fewer nodules/tissue section.

5 Transient transfection with pcDNA3/sflt-1 (n=7), as compared to a pcDNA control (n=6), resulted in slower growing tumor nodules on all days examined ($p < 0.01$). These cells had identical growth rates in vitro over a period of 96 hours. Average nodule volumes for sflt-1 transfected cells were 50 mm³, 75 mm³ and 190 mm³ on days 7,
10 12, and 17, respectively. In contrast, using control pcDNA3 transfected cells, nodules were 151 mm³, 261 mm³ and 474 mm³ on days 7, 12 and 17. Similarly, mean lung weights were less in animals receiving pcDNA3/sflt-1 (171 mg, n=3) transfected cells by tail vein injection compared to pcDNA3 controls (205 mg, n=3). Figure 4 shows a marked
15 decrease in tumor volume in nude mice injected with HT-1080 cells which transiently express sVEGF-RI in the form of pcDNA3/sflt-1.

 A second study designed to investigate the ability of sFLT-based gene therapy to be applied to treatment of tumor metastasis
20 yielded similar results. HT-1080 cells were transiently transfected with pcDNA3 or pcDNA3/sflt-1. 4×10^6 cells were injected at day 0 via the tail vein of each mouse. The animals were sacrificed after one month and the lungs were extracted, weighed, and examined histologically for tumor burden. Lung histology performed on animals receiving
25 intravenously injected tumors revealed a striking difference between the two groups. pcDNA3 transfected cells were associated with pulmonary intramural tumor spread, massive parenchymal edema and mononuclear infiltrate 20 days after intravenous injection of tumor cells. In contrast, pcDNA3/sflt-1 transfected cells were associated with
30 rare tumor foci, the absence of edema and almost normal lung parenchyma histology. Eight of 9 animals injected with HT-1080 cells transiently expressing sVEGF-RI were clear of tumor growth. Conversely, HT-1080 tumor cells transfected with the pcDNA3 control plasmid showed 2 of 9 without tumor growth while 7 of 9 formed lung

nodules. This data shows that sFLT-based gene therapy applications may be utilized to treat tumor metastasis.

5 Third, a syngeneic model was examined. Pooled clones were generated for either pcDNA3 or pcDNA3/sflt-1 in GL261 mouse glioma cells. Cell counting of the cells grown in culture revealed no differences between the groups. All 3 pcDNA3 animals grew large tumors after approximately one month. Two of 3 in the sFLT-1 group were tumor free. The third had formed a very small tumor. The histopathology differed but all tumors had a clear malignant appearance.

EXAMPLE 3

Inhibition of Tumor Angiogenesis in Mice by Administration of Cells Stably Transfected with a cDNA Fragment Encoding sVEGF-RI

15 The study described in Example 2 was repeated with HT-1080 cells stably transfected with either the pcDNA3 control of pcDNA3/sflt-1. Figure 5 shows a virtually complete inhibition of tumor growth compared to the additional data generated with transiently transfected tumor cells.

20 To determine the effects of sVEGF-RI on animals survival, the human glioma cell line D-54MG was stably transfected with pcDNA3/psflt-1 or a pcDNA3 control. Clones were pooled and the same number of cells were injected intracranially using a mouse stereotactic device with skull sutures as landmarks. The model has previously been determined to have reliable survival characteristics. Animals were treated identically post-operatively. Figure 6 shows that mice injected with an untransfected control died by day 26, mice injected with pcDNA3 transfected control cells died by day 25, wherein all mice which received pcDNA3/sVEGF-R transfected cells were alive at day 41. Figure 7 shows extended data points from this experiment, showing that the mean survival for D-54MG cells transfected with pcDNA3/sflt-1 was 46.5 days. As noted earlier in this paragraph, D54-MG human glioma cells were transfected with pcDNA3/sflt-1 or pcDNA3 using AdpL transfection.

The cells were subsequently propagated in complete medium containing 400 µg/mL of G418 antibiotic (Gibco BRL, Grand Island, NY) for one month to select for a population of clones that contained the pcDNA3/sflt-1 or pcDNA3 plasmid. The selected cells, representing a population of

5 pooled clones were then harvested using trypsin/EDTA solution (Gibco) and counted using a hemacytometer with trypan blue exclusion. The cells were resuspended to a final concentration of 10^7 cells/100 µL in serum-free DMEM/F12 containing 5% methylcellulose as a vehicle to enhance cell viability. A midline scalp incision was made, followed by a

10 0.5 mm burr hole 1.5-2.0 mm to the right of the midline and 0.5-1.0 mm posterior to the coronal suture. The cells were loaded into a 100 µL microsyringe and 5 µL was injected stereotactically. A 30 gauge needle mounted on the microsyringe was inserted vertically through the burr hole to a depth of 2.5 mm. Forty-five to sixty seconds after injection, the

15 needle was slowly withdrawn and the incision closed with 9 mm Michel wound clips. Mice were returned to sterile microisolator polycarbonate cages, placed over a heating pad until recovery, and provided autoclaved lab chow and sterile water ad libitum. Animals were assessed twice daily for survival. These results demonstrate that sFLT-1 animals

20 survived longer than historical controls and subsequent controls.

EXAMPLE 4

Construction of AdHCMVsFLT-1

25 Several systems have been developed for the construction of helper-independent adenovirus (Ad) vectors and have been recently been reviewed by Graham and Prevec (1995, *Mol. Biotech.* 3: 207-220) and Hitt et al. (1995, Techniques for human adenovirus vector construction and characterization, In *Methods in Molecular Genetics, Volume 7*.

30 *Molecular Virology Techniques Part B*, ed. Kenneth W. Adolph, Academic Press, Inc. Orlando, Florida). All of these systems involve cloning the transgene of interest (coding region flanked by appropriate regulatory sequences) into a shuttle plasmid in which it is flanked by Ad sequences homologous to the region of the viral genome into which the

transgene will be introduced. The DNA from the shuttle plasmid is then rescued into virus by either direct ligation *in vitro* followed by transfection or by *in vivo* homologous recombination following transfection into 293 cells.

5 E1 shuttle plasmids have been developed for the rescue of inserts into the E1 region. These plasmids contain the left 16% of the Ad genome with a deletion of E1 sequences and cloning sites into which the transgene is introduced. If convenient restriction sites are available in the vector backbone, direct ligation of the shuttle plasmid to purified
10 viral DNA can be performed *in vitro* followed by transfection into 293 cells to generate infectious virus. This method although efficient can require extensive screening if the viral DNA is not completely restricted and in many cases is not practical due to the lack of unique correctly positioned restriction sites. For these reasons many protocols rely on *in*
15 *vivo* homologous recombination to generate infectious virus. To construct a virus by homologous recombination the shuttle plasmid can be transfected into 293 cells with purified viral DNA that has been restricted in the left end or with viral DNA contained in a second plasmid. As with direct ligation the use of purified viral DNA
20 sometimes requires extensive screening to obtain the desired vector because of the regeneration of parental virus and for this reason plasmid systems are more desirable. A number of plasmid systems have been developed for rescuing inserts into E1 (McGrory et al., 1988, *Virology* 163: 614-6170) or E3 (Ghosh-Choudhury, et al., 1986, *Gene* 50:
25 161-171; Mittal, et al., 1993, *Virus Res.* 28: 67-90) or both (Bett et al., 1994, *Proc. Natl. Acad. Sci. USA* 91: 8802-8806) regions.

 The steps involved in the construction of the helper independent Ad vectors expressing sFLT-1 are outlined below. All steps involve the use of standard protocols for generating adenovirus vectors
30 (Hitt, et al., 1995, In *Methods in Molecular Genetics, Volume 7. Molecular Virology Techniques Part B*, ed. Kenneth W. Adolph, Academic Press, Inc. Orlando, FL.). The coding sequences for sFLT were obtained from plasmid psflt-1 by BamHI digestion and inserted into the BamHI site in the polycloning region of E1 shuttle plasmid

pΔE1sp1HCMV-BGHpA, generating pHCMVsFLT-1. pΔE1sp1HCMV-BGHpA contains Ad5 sequences from bp 1 to 341 and bp 3524 to 5790 with a promoter cassette consisting of the HCMV promoter, a polycloning region and the Bovine growth hormone polyadenylation signal inserted in the E1 anti parallel orientation between Ad5 bp 341 and bp 3524.

pHCMVsFLT-1 was then cotransfected into 293 cells with Ad genome plasmid pJM17 (McGrory, et al., 1988, *Virology* 163:614-617) and virus AdHCMVsFLT-1 was generated by *in vivo* recombination between the plasmids. pJM17 contains essentially the entire Ad genome but is non infectious in single transfections of 293 cells since it contains an insertion of a pBR322 derivative at bp 1339 in Ad5 sequences which makes the resulting viral genome too large to package. *In vivo* recombination between pJM17 and pHCMVsFLT-1 generates a vector of a packagable size containing the sFLT-1 expression cassette in the E1 region.

An additional recombinant adenoviral virus is also disclosed. It is essentially the same as the vector described above but utilizes a slightly different HCMV promoter segment consisting of the HCMV promoter and first intron (Intron A). This construct increases expression levels within the mammalian host. To construct this vector sFLT-1 coding sequences were obtained from plasmid pHCMVsFLT-1 (described above) by digestion with KpnI and EcoRI. The sFLT-1 fragment was then inserted into the KpnI and EcoRI sites in E1 shuttle plasmid pHCMVI1-BGHpA, generating pHCMVI1sFLT-1.

pHCMVI1sFLT-1 has been cotransfected into 293 cells with Ad genome plasmid pJM17. Alternatively, pHCMVI1sFLT-1 was digested with PacI and ligated with purified viral DNA from the virus AdDE1PacIE3 also digested with PacI. Following the transfection of the ligation products into 293 cells viral plaques were screened to obtain the vector AdHCMVI1sFLT-1.

EXAMPLE 5

Stable Transfection of Human HT1080 Fibrosarcoma Cells with sFlt-1
Inhibits Solid Tumor Growth

5 *Generation of sFlt-1 plasmid-* An additional plasmid
(pcDNAIAsFLT-1) was constructed that contained the HCMV Intron-A
upstream to the sflt-1 cDNA in order to generate HT-1080 clones that
secrete increased amounts of sflt-1. This intron has been demonstrated
10 in previous studies to enhance gene expression by 10-100 fold above
plasmids containing the HCMV early promoter alone. For the
construction of pcDNAIAsFLT-1, pcDNA3 was digested with NruI and
KpnI (to remove the HCMV promoter) and ligated with the MscI/KpnI
fragment from plasmid pVIJNS-MCS (containing the HCMV promoter
and Intron A), generating pcDNAINTA. pcDNAINTA was then
15 digested with KpnI and EcoRI and ligated to a KpnI/EcoRI fragment
containing the sFLT-1 coding sequences, generating pcDNAIAsFLT-1.

Selection of HT1080 clones stably transfected with
pcDNAIAsFLT-1 and expressing sFlt-1 - Human fibrosarcoma HT1080
tumor cells (Rasheed *et al.*, 1974, *Cancer* 33:1027-1033) were transfected
20 with the plasmid (pcDNAIAsFLT-1) containing the human sFlt-1 gene
under the control of the HCMV promoter containing the first HCMV
intron and the selectable G418 drug resistance gene. Pooled stably
transfected HT1080 cells were plated in 100 cm dishes at a density of 10
and 100 cells/plate. The cells were grown in DMEM supplemented
25 medium [Dulbecco's Modified Eagle Medium/F-12 (DMEM), GIBCOBRL
(Cat# 11331-030), 10% fetal bovine serum, (GIBCOBRL Cat# 16000-028)
and 1 X penicillin-streptomycin, (GIBCOBRL Cat# 15070-063)] with 500
µg/ml of G418 (GIBCOBRL Cat# 10131-035). The medium was replaced
every other day until individual colonies grew to diameters of
30 approximately 2.5 mm. Isolated colonies were treated with trypsin
(GIBCOBRL Cat# 25200-056), transferred to 24 well plates and grown to
confluence. One ml of medium was removed and tested for VEGF
binding activity. The stable clone chosen for further studies had similar
growth rates *in vitro* compared to both untransfected cells and cells

transfected with pCDNA3, with cell division occurring approximately every 48 hours.

VEGF Binding Protocol - Heparin-Sepharose CL-6B

(Pharmacia Cat# 17-0467-01) was washed 3 times with phosphate buffered saline [PBS] (GIBCOBRL Cat# 20012-027), and resuspended in an equal volume of PBS. One ml of conditioned medium was removed from each well, mixed with 50 μ l of the heparin-Sepharose CL-6B slurry and incubated overnight at 4 °C with constant mixing. The heparin-Sepharose beads were pelleted by centrifugation (10,000 x g for 2 min) and washed 3 times with PBS. Bound protein was eluted with 40 μ l of PBS containing 1.2 M NaCl. A 10 μ l aliquot was removed and added to 10 μ l of DMEM/0.2% gelatin, 1 μ l of 125 I-VEGF (Amersham Cat# IM 274; 100,000 cpm/ μ l) was added and incubated for 20 min at room temperature. Two μ l of 10 mM BS³ bis(sulfosuccinimidyl) suberate [BS³], (Pierce Cat# 21579 G) was added to the reaction and incubated for an additional 15 min at room temperature. The crosslinking reaction was stopped by the addition of 20 μ l of 2X Laemmli sample buffer (BioRad Cat# 161-0737). Crosslinked complexes were separated by SDS/7.5% PAGE and visualized by autoradiography.

Preparation of selected clones for the tumor growth study -
Cells were plated in T-75 flasks and grown to confluence in DMEM supplemented medium. Cells were washed with PBS and trypsinized in 2 ml. Trypsinization was stopped by the addition of 8 ml DMEM supplemented medium and the detached cells were removed and counted. The cells were pelleted by centrifugation (1000 rpm in a Sorvall 6000B table top centrifuge) for 5 min and resuspended in PBS with calcium and magnesium at a final concentration of 1.0×10^7 cells/ml and 0.5 ml of cells was injected subcutaneously into mice.

Results - HT-1080 cells (0.5×10^6 cells/0.5 ml) stably transfected with either control plasmid or plasmid encoding sflt-1 [pcDNA1AsFLT-1] (n = 10/group) that was cloned and selected for high sflt-1 expression were injected subcutaneously into Balb/c nu/nu female mice (Charles River Laboratories). Tumor length and width were

measured as a function of time and used to calculate tumor volume by the equation:

$$\text{Volume} = 4/3 \cdot \pi \cdot ((\text{length}/2)(\text{width}/2)(\text{length} + \text{width})/4),$$

which estimates the volume of half a prolate ellipsoid assuming that the height is the average of the length and width. On day 12 after implantation tumor ulceration was visible so the tumors were removed and weighed; expression of sFlt-1 caused a 93% reduction in tumor mass. As shown in Figure 8, tumor growth as measured by average volume and mass (\pm SD) were significantly inhibited by expression of the sflt-1 gene (1-tailed Student's t-test, $p < 0.0001$ for comparison of masses).

EXAMPLE 6

Infection of Human HT1080 Fibrosarcoma Cells with Replication-Defective Adenovirus Expressing Human sFlt-1 Inhibits Tumor Growth

Generation of sFlt-1 adenoviral constructs are as described in Example Section 4.

Adenoviral infection of HT1080 cells in vitro and implantation in vivo - Cells were plated in T-75 flasks and grown to confluence in DMEM supplemented medium. One flask of cells was trypsinized (2 ml), the cells were removed and resuspended in DMEM supplemented medium and counted to determine the number of cells/plate. Growth medium was removed from flasks and the attached cells were washed with PBS containing calcium and magnesium. Either control adenovirus or adenovirus expressing human sFlt-1 under control of HCMV/intron A were added to flasks at an multiplicity of infection of 20 virus pfu (plaque forming units)/cell in 2 ml of PBS with calcium and magnesium and incubated for 1 hr at 37 °C. The virus was removed and the cells were incubated in a humidified incubator with 5% CO₂ at 37 °C for an additional 24 hr. Cells were washed with PBS and trypsinized with 2 ml of trypsin. Trypsinization was stopped by the addition of DMEM supplemented medium and the detached cells were

removed and counted. The cells were pelleted by centrifugation (1000 rpm in a Sorvall 6000B table top centrifuge) for 5 min and resuspended in PBS with calcium and magnesium at a final concentration of 1.0×10^7 cells/ml and 0.5 ml of cells was injected subcutaneously into 6-8 week old Balb/c nu/nu female mice.

Results - Tumor cells that were exposed to either no virus, a control adenovirus or adenovirus expressing sFlt-1 under control of the HCMV/intron A promoter [AdHCMVI1sflt-1] (n = 5/group) were allowed to grow subcutaneously in nude mice. After 11 days of *in vivo* growth the skin over the tumor began to ulcerate in control animals so the tumors were removed from all animals and weighed. The mean group tumor masses \pm SEMs are shown in Figure 9.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: MERCK & CO., INC.

(ii) TITLE OF THE INVENTION: GENE THERAPY FOR INHIBITION OF
ANGIOGENESIS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: J. Mark Hand - MERCK & CO., INC.
(B) STREET: 126 EAST LINCOLN AVENUE - P.O. BOX 2000
(C) CITY: RAHWAY
(D) STATE: NJ
(E) COUNTRY: US
(F) ZIP: 07065-0900

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: U.S. 60/026,641
(B) FILING DATE: September 24, 1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Hand, J. Mark
(B) REGISTRATION NUMBER: 36,545
(C) REFERENCE/DOCKET NUMBER: 19810Y

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 908-594-3905
(B) TELEFAX: 908-594-4720
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2313 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE:

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	GCGGACACTC CTCTCGGCTC CTCCCCGGCA GCGGCGGCGG CTCGGAGCGG GCTCCGGGGC	60
	TGGGGTGCAG CGGCCAGCGG GCCTGGCGGC GAGGATTACC CGGGGAAGTG GTTGCTCCTT	120
15	GGCTGGAGCC GCGAGACGGG CGCTCAGGGC GCGGGGCCGG CGGCGGCGAA CGAGAGGACG	180
	GACTCTGGCG GCCGGGTCGT TGGCCGGGGG AGCGCGGGCA CCGGGCGAGC AGGCCCGCTC	240
	GCGCTCACCA TGGTCAGCTA CTGGGACACC GGGGTCCTGC TGTGCGCGCT GCTCAGCTGT	300
	CTGCTTCTCA CAGGATCTAG TTCAGGTTCA AAATTAAAAG ATCCTGAACT GAGTTTAAAA	360
	GGCACCCAGC ACATCATGCA AGCAGGCCAG AACTGTCATC TCCAATGCAG GGGGAAGCA	420
20	GCCCCATAAT GGTCTTTGCC TGAAATGGTG AGTAAGGAAA GCGAAAGGCT GAGCATAACT	480
	AAATCTGCCT GTGGAAGAAA TGGCAAACAA TTCTGCAGTA CTTTAACCTT GAACACAGCT	540
	CAAGCAAACC AACTGGCTT CTACAGCTGC AAATATCTAG CTGTACCTAC TTCAAAGAAG	600
	AAGGAAACAG AATCTGCAAT CTATATATTT ATTAGTGATA CAGGTAGACC TTTCGTAGAG	660
	ATGTACAGTG AAATCCCCGA AATTATACAC ATGACTGAAG GAAGGGAGCT CGTCATTTCCC	720
25	TGCCGGGTTA CGTCACCTAA CATCACTGTT ACTTTAAAAA AGTTTCCACT TGACACTTTG	780
	ATCCCTGATG GAAAACGCAT AATCTGGGAC AGTAGAAAGG GCTTCATCAT ATCAAATGCA	840
	ACGTACAAAG AAATAGGGCT TCTGACCTGT GAAGCAACAG TCAATGGGCA TTTGTATAAG	900
	ACAAACTATC TCACACATCG ACAAACCAAT ACAATCATAG ATGTCCAAAT AAGCACACCA	960
	CGCCCAGTCA AATTACTTAG AGGCCATACT CTTGTCCTCA ATTGTACTGC TACCACTCCC	1020
30	TTGAACACGA GAGTTCAAAT GACCTGGAGT TACCCTGATG AAAAAAATAA GAGAGCTTCC	1080
	GTAAGGCGAC GAATTGACCA AAGCAATTCC CATGCCAACA TATTCTACAG GTTCTTACT	1140
	ATTGACAAAA TGCAGAACAA AGACAAAGGA CTTTATACTT GTCGTGTAAG GAGTGGACCA	1200
	TCATTCAAAT CTGTTAACAC CTCAGTGCAT ATATATGATA AAGCATTCAT CACTGTGAAA	1260
	CATCGAAAAC AGCAGGTGCT TGAAACCGTA GCTGGCAAGC GGTCTTACCG GCTCTCTATG	1320
35	AAAGTGAAGG CATTTCCCTC GCCGGAAGTT GTATGGTTAA AAGATGGGTT ACCTGCGACT	1380
	GAGAAATCTG CTCGCTATTT GACTCGTGGC TACTCGTTAA TTATCAAGGA CGTAACTGAA	1440
	GAGGATGCAG GGAATTATAC AATCTTGCTG AGCATAAAAC AGTCAAATGT GTTTAAAAAC	1500
	CTCACTGCCA CTCTAATTGT CAATGTGAAA CCCCAGATTT ACGAAAAGGC CGTGTCTATC	1560

TTTCCAGACC CGGCTCTCTA CCCACTGGGC AGCAGACAAA TCCTGACTTG TACCGCATAT 1620
 GGTATCCCTC AACCTACAAT CAAGTGGTTC TGGCACCCCT GTAACCATAA TCATTCCGAA 1680
 GCAAGGTGTG ACTTTTGTTC CAATAATGAA GAGTCCTTTA TCCTGGATGC TGACAGCAAC 1740
 ATGGGAAACA GAATTGAGAG CATCACTCAG CGCATGGCAA TAATAGAAGG AAAGAATAAG 1800
 5 ATGGCTAGCA CCTTGGTTGT GGCTGACTCT AGAATTTCTG GAATCTACAT TTGCATAGCT 1860
 TCCAATAAAG TTGGGACTGT GGAAGAAAC ATAAGCTTTT ATATCACAGA TGTGCCAAAT 1920
 GGGTTTCATG TTAAGTTGGA AAAAATGCCG ACGGAAGGAG AGGACCTGAA ACTGTCTTGC 1980
 ACAGTTAACA AGTTCTTATA CAGAGACGTT ACTTGGATTT TACTGCGGAC AGTTAATAAC 2040
 AGAACAATGC ACTACAGTAT TAGCAAGCAA AAAATGGCCA TCACTAAGGA GCACTCCATC 2100
 10 ACTCTTAATC TTACCATCAT GAATGTTTCC CTGCAAGATT CAGGCACCTA TGCCTGCAGA 2160
 GCCAGGAATG TATACACAGG GGAAGAAATC CTCCAGAAGA AAGAAATTAC AATCAGAGGT 2220
 GAGCACTGCA ACAAAAAGGC TGTTTTCTCT CGGATCTCCA AATTTAAAAG CACAAGGAAT 2280
 GATTGTACCA CACAAAGTAA TGTAACAT TAA 2313

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 687 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1 5 10 15
 Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
 20 25 30
 35 Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
 35 40 45
 Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
 50 55 60
 40 Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
 65 70 75 80

	Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
	85 90 95
	Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
	100 105 110
5	Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
	115 120 125
	Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
	130 135 140
	Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
10	145 150 155 160
	Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
	165 170 175
	Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
	180 185 190
15	Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
	195 200 205
	Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
	210 215 220
	Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
20	225 230 235 240
	Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
	245 250 255
	Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
	260 265 270
25	Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
	275 280 285
	Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
	290 295 300
	Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
30	305 310 315 320
	Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Phe Ile Thr Val
	325 330 335
	Lys His Arg Lys Gln Gln Val Leu Glu Thr Val Ala Gly Lys Arg Ser
	340 345 350

Tyr Arg Leu Ser Met Lys Val Lys Ala Phe Pro Ser Pro Glu Val Val
 355 360 365
 Trp Leu Lys Asp Gly Leu Pro Ala Thr Glu Lys Ser Ala Arg Tyr Leu
 370 375 380
 5 Thr Arg Gly Tyr Ser Leu Ile Ile Lys Asp Val Thr Glu Glu Asp Ala
 385 390 395 400
 Gly Asn Tyr Thr Ile Leu Leu Ser Ile Lys Gln Ser Asn Val Phe Lys
 405 410 415
 Asn Leu Thr Ala Thr Leu Ile Val Asn Val Lys Pro Gln Ile Tyr Glu
 10 420 425 430
 Lys Ala Val Ser Ser Phe Pro Asp Pro Ala Leu Tyr Pro Leu Gly Ser
 435 440 445
 Arg Gln Ile Leu Thr Cys Thr Ala Tyr Gly Ile Pro Gln Pro Thr Ile
 450 455 460
 15 Lys Trp Phe Trp His Pro Cys Asn His Asn His Ser Glu Ala Arg Cys
 465 470 475 480
 Asp Phe Cys Ser Asn Asn Glu Glu Ser Phe Ile Leu Asp Ala Asp Ser
 485 490 495
 Asn Met Gly Asn Arg Ile Glu Ser Ile Thr Gln Arg Met Ala Ile Ile
 20 500 505 510
 Glu Gly Lys Asn Lys Met Ala Ser Thr Leu Val Val Ala Asp Ser Arg
 515 520 525
 Ile Ser Gly Ile Tyr Ile Cys Ile Ala Ser Asn Lys Val Gly Thr Val
 530 535 540
 25 Gly Arg Asn Ile Ser Phe Tyr Ile Thr Asp Val Pro Asn Gly Phe His
 545 550 555 560
 Val Asn Leu Glu Lys Met Pro Thr Glu Gly Glu Asp Leu Lys Leu Ser
 565 570 575
 Cys Thr Val Asn Lys Phe Leu Tyr Arg Asp Val Thr Trp Ile Leu Leu
 30 580 585 590
 Arg Thr Val Asn Asn Arg Thr Met His Tyr Ser Ile Ser Lys Gln Lys
 595 600 605
 Met Ala Ile Thr Lys Glu His Ser Ile Thr Leu Asn Leu Thr Ile Met
 610 615 620

	Asn	Val	Ser	Leu	Gln	Asp	Ser	Gly	Thr	Tyr	Ala	Cys	Arg	Ala	Arg	Asn	
	625					630					635					640	
	Val	Tyr	Thr	Gly	Glu	Glu	Ile	Leu	Gln	Lys	Lys	Glu	Ile	Thr	Ile	Arg	
					645					650						655	
5	Gly	Glu	His	Cys	Asn	Lys	Lys	Ala	Val	Phe	Ser	Arg	Ile	Ser	Lys	Phe	
					660					665						670	
	Lys	Ser	Thr	Arg	Asn	Asp	Cys	Thr	Thr	Gln	Ser	Asn	Val	Lys	His		
					675					680						685	

10

WHAT IS CLAIMED IS:

1. A method of inhibiting angiogenesis of a solid or metastatic tumor in a mammalian host which comprises delivering a DNA vector to said mammalian host, said DNA vector expressing a soluble form of a tyrosine kinase receptor which forms a dimer with VEGF, a VEGF homologue or a VEGF-specific tyrosine kinase receptor protein.
2. The method of claim 1 wherein said mammalian host is a human.
3. The method of claim 2 wherein said DNA vector is a recombinant adenovirus.
4. The method of claim 2 wherein said DNA vector is a recombinant DNA plasmid vector.
5. The method of claim 3 wherein said recombinant adenovirus is delivered by infection into cells a solid tumor or cells adjacent to said solid tumor.
6. The method of claim 4 wherein said cells are selected from the group consisting of adipose cells, muscle cells and vascular endothelial cells.
7. A method of inhibiting solid tumor angiogenesis or metastatic tumor angiogenesis in a mammalian host which comprises delivering a DNA vector to said mammalian host, said DNA vector expressing a soluble form of FLT-1 which forms a dimer with VEGF, a VEGF homologue or a VEGF-specific tyrosine kinase receptor protein.
8. The method of claim 7 wherein said mammalian host is a human.

9. The method of claim 8 wherein said DNA vector is a recombinant adenovirus.

5 10. The method of claim 8 wherein said DNA vector is a recombinant DNA plasmid vector.

10 11. The method of claim 9 wherein said recombinant adenovirus is delivered by infection into cells of a solid tumor or cells adjacent to said solid tumor.

12. The method of claim 11 wherein said adjacent escent cells are selected from the group consisting of adipose cells, muscle cells and vascular endothelial cells.

15 13. The method of claim 12 wherein said recombinant adenovirus AdHCMVsFLT-1.

20 14. The method of claim 12 wherein said recombinant adenovirus AdHCMVI1sFLT.

25 15. The method of claim 10 wherein said recombinant DNA plasmid vector is delivered by injection into cells of a solid tumor or cells adjacent to said solid tumor.

16. The method of claim 15 wherein said quiescent cells are selected from the group consisting of adipose cells, muscle cells and vascular endothelial cells.

30 17. The method of claim 16 wherein said recombinant DNA plasmid vector is pcDNA3/sflt-1.

18. The method of claim 16 wherein said recombinant DNA plasmid vector is pcDNA3IA/sflt-1.

19. A recombinant virus comprising a DNA fragment encoding a soluble form of a VEGF receptor which forms a dimer with VEGF, a VEGF homologue or a VEGF-specific tyrosine kinase receptor protein said recombinant vector containing at least one regulatory sequence which controls expression of said DNA fragment within a mammalian host.

20. A recombinant virus of claim 19 which is a recombinant adenovirus.

21. A recombinant virus of claim 20 wherein said DNA fragment encodes a soluble VEGF receptor, sFLT-1 .

22. A recombinant virus of claim 21 wherein said DNA fragment encodes a human sFLT-1 VEGF receptor as set forth in SEQ ID NO:2.

23. The recombinant virus of claim 22 which is AdHCMVsFLT-1.

24. The method of claim 22 wherein said recombinant adenovirus AdHCMV1sFLT.

25. A method of determining efficacy of inhibiting tumor angiogenesis, which comprises:

(a) transfecting cultured tumor cells with a DNA vector expressing sFLT;

(b) injecting said transfected tumor cells into a mouse;

(c) sacrificing said mouse after an interval allowing for tumor growth within said mouse; and,

(d) observing formation of tumor nodules is
5 said mouse as compared to a mouse injected with tumor cells transfected with vector along or untransfected tumor cells.

1/8

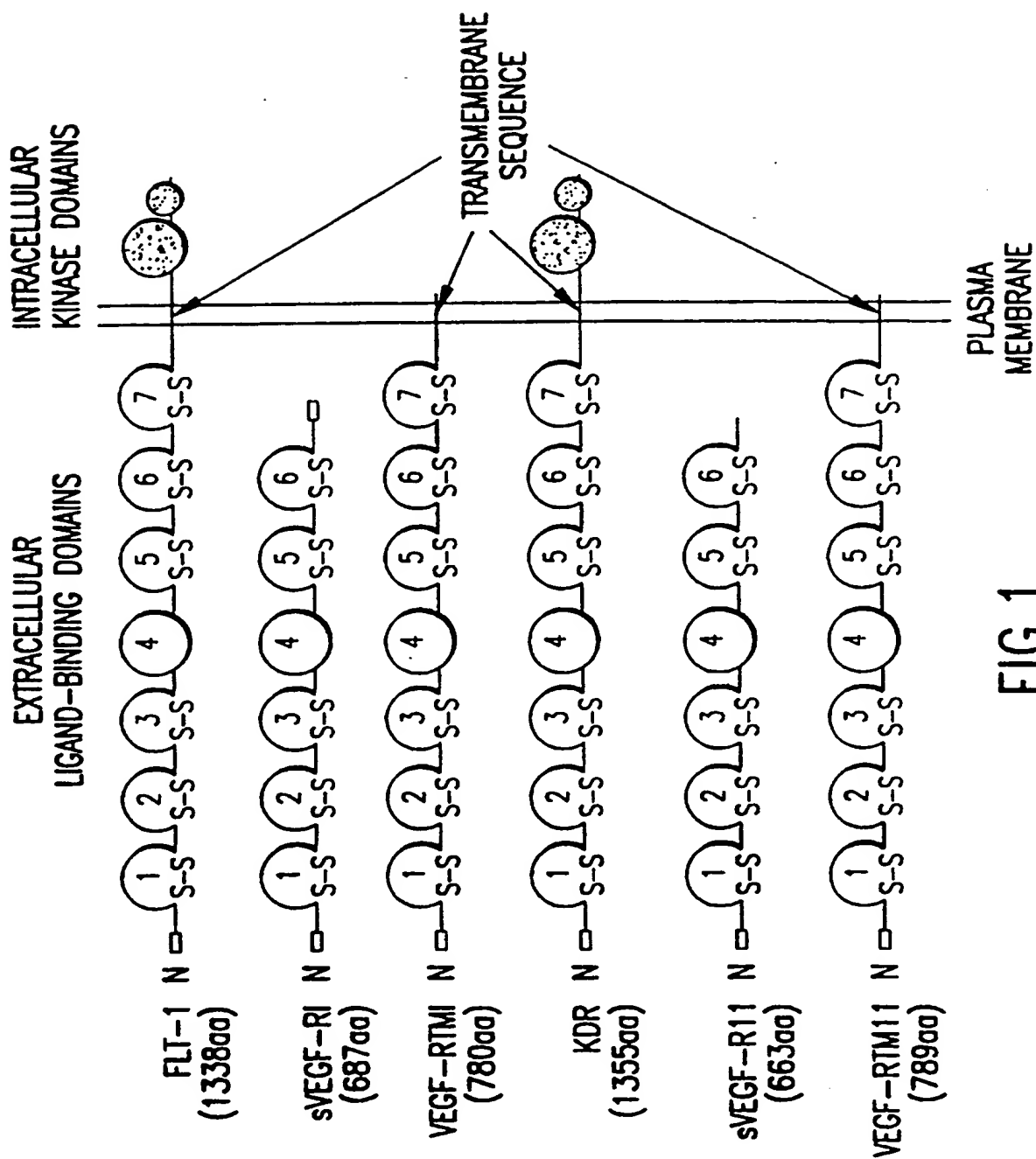


FIG. 1

2/8

GCGGACACTC	CTCTCGGCTC	CTCCCCGGCA	GCGGCGGCGG	CTCGGAGCGG	GCTCCGGGGC	60
TCGGGTGCAG	CGGCCAGCGG	GCCTGGCGGC	GAGGATTACC	CGGGGAAGTG	GTTGTCTCCT	120
GGCTGGAGCC	GCGAGACGGG	CGCTCAGGGC	GCGGGGCCGG	CGGCGGCGAA	CGAGAGGACG	180
GACTCTGGCG	GCCGGGTCGT	TGGCCGGGGG	AGCGCGGGCA	CCGGGCGAGC	AGGCCGCGTC	240
GCGCTACCA	TGGTCAGCTA	CTGGGACACC	GGGGTCCTGC	TGTGCGCGCT	GCTCAGCTGT	300
CTGCTTCTCA	CAGGATCTAG	TTCAGGTTCA	AAATTAAG	ATCCTGAACT	GAGTTTAAAA	360
GGCACCCAGC	ACATCATGCA	AGCAGGCCAG	ACACTGCATC	TCCAATGCAG	GGGGGAAGCA	420
GCCCATAAAT	GGTCTTTGCC	TGAAATGGTG	AGTAAGGAAA	GCGAAAGGCT	GAGCATAACT	480
AAATCTGCCT	GTGGAAGAAA	TGGCAAACAA	TTCTGCAGTA	CTTTAACCTT	GAACACAGCT	540
CAAGCAAACC	ACACTGGCTT	CTACAGCTGC	AAATATCTAG	CTGTACCTAC	TTCAAAGAAG	600
AAGGAAACAG	AATCTGCAAT	CTATATATTT	ATTAGTGATA	CAGGTAGACC	TTTCGTAGAG	660
ATGTACAGTG	AAATCCCCGA	AATTATACAC	ATGACTGAAG	GAAGGGAGCT	CGTCATTCCC	720
TGCCGGGTTA	CGTCACCTAA	CATCACTGTT	ACTTTAAAAA	AGTTTCCACT	TGACACTTTG	780
ATCCCTGATG	GAAAACGCAT	AATCTGGGAC	AGTAGAAAGG	GCTTCATCAT	ATCAAATGCA	840
ACGTACAAAG	AAATAGGGCT	TCTGACCTGT	GAAGCAACAG	TCAATGGGCA	TTTGTATAAG	900
ACAAACTATC	TCACACATCG	ACAAACCAAT	ACAATCATAG	ATGTCCAAAT	AAGCACACCA	960
CGCCCAGTCA	AATTACTTAG	AGGCCATACT	CTTGTCTCTA	ATTGTACTGC	TACCACTCCC	1020
TTGAACACGA	GAGTTCAAAT	GACCTGGAGT	TACCCTGATG	AAAAAATAA	GAGAGCTTCC	1080
GTAAGGCGAC	GAATTGACCA	AAGCAATTCC	CATGCCAACA	TATTCTACAG	TGTTCTTACT	1140
ATTGACAAAA	TGCAGAACAA	AGACAAAGGA	CTTTATACTT	GTCGTGTAAG	GAGTGGACCA	1200
TCATTCAAAT	CTGTTAACAC	CTCAGTGCAT	ATATATGATA	AAGCATTTCAT	CACTGTGAAA	1260
CATCGAAAAC	AGCAGGTGCT	TGAAACCGTA	GCTGGCAAGC	GGTCTTACCG	GCTCTCTATG	1320
AAAGTGAAGG	CATTTCCCTC	GCCGGAAGTT	GTATGGTTAA	AAGATGGGTT	ACCTGCGACT	1380
GAGAAATCTG	CTCGCTATTT	GACTCGTGGC	TACTCGTTAA	TTATCAAGGA	CGTAACTGAA	1440
GAGGATGCAG	GGAATTATAC	AATCTTGCTG	AGCATAAAAC	AGTCAAATGT	GTTTAAAAAC	1500
CTCACTGCCA	CTCTAATTGT	CAATGTGAAA	CCCCAGATTT	ACGAAAAGGC	CGTGTCTATG	1560
TTTCAGACC	CGGCTCTCTA	CCCACTGGGC	AGCAGACAAA	TCCTGACTTG	TACCGCATAT	1620
GGTATCCCTC	AACCTACAAT	CAAGTGGTTC	TGGCACCCCT	GTAACCATAA	TCATTCCGAA	1680
GCAAGGTGTG	ACTTTTGTTT	CAATAATGAA	GAGTCCTTTA	TCCTGGATGC	TGACAGCAAC	1740
ATGGGAAACA	GAATTGAGAG	CATCACTCAG	CGCATGGCAA	TAATAGAAGG	AAAGAATAAG	1800
ATGGCTAGCA	CCTTGGTTGT	GGCTGACTCT	AGAATTTCTG	GAATCTACAT	TTGCATAGCT	1860
TCCAATAAAG	TTGGGACTGT	GGGAAGAAAC	ATAAGCTTTT	ATATCACAGA	TGTGCCAAAT	1920
GGGTTTCATG	TTAACTTGGA	AAAAATGCCG	ACGGAAGGAG	AGGACCTGAA	ACTGTCTTGC	1980
ACAGTTAACA	AGTTCTTATA	CAGAGACGTT	ACTTGGATTT	TACTGCGGAC	AGTTAATAAC	2040
AGAACAATGC	ACTACAGTAT	TAGCAAGCAA	AAAATGGCCA	TACTAAGGA	GCACTCCATC	2100
ACTCTTAATC	TTACCATCAT	GAATGTTTCC	CTGCAAGATT	CAGGCACCTA	TGCCTGCAGA	2160
GCCAGGAATG	TATACACAGG	GGAAGAAATC	CTCCAGAAGA	AAGAAATTAC	AATCAGAGGT	2220
GAGCACTGCA	ACAAAAAGGC	TGTTTTCTCT	CGGATCTCCA	AATTTAAAAG	CACAAGGAAT	2280
GATTGTACCA	CACAAAGTAA	TGTAAAACAT	TAA			2313

FIG.2
SUBSTITUTE SHEET (RULE 28)

3/8

Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1 Cys Leu Leu 20 Thr Gly Ser Ser 25 Gly Ser Lys Leu 30 Lys Asp Pro
 Glu Leu Ser 35 Leu Lys Gly Thr 40 His Ile Met Gln 45 Ala Gly Gln Thr
 Leu His 50 Leu Gln Cys Arg Gly 55 Glu Ala Ala His Lys 60 Trp Ser Leu Pro
 Glu Met Val Ser Lys 70 Glu Ser Glu Arg Leu Ser 75 Ile Thr Lys Ser Ala
 65 Cys Gly Arg Asn 85 Gly Lys Gln Phe Cys 90 Thr Leu Thr Leu Asn 80 Thr
 Ala Gln Ala Asn 100 His Thr Gly Phe Tyr 105 Ser Cys Lys Tyr Leu 95 Ala Val
 Pro Thr Ser 115 Lys Lys Lys Glu 120 Thr Glu Ser Ala Ile Tyr 110 Phe Ile
 Ser Asp 130 Thr Gly Arg Pro Phe 135 Val Glu Met Tyr Ser 140 Glu Ile Pro Glu
 Ile Ile His Met Thr Glu 150 Gly Arg Glu Leu Val 155 Ile Pro Cys Arg Val
 145 Thr Ser Pro Asn 165 Thr Val Thr Leu Lys 170 Phe Pro Leu Asp 160 Thr
 Leu Ile Pro Asp 180 Gly Lys Arg Ile 185 Trp Asp Ser Arg Lys Gly Phe
 Ile Ile Ser 195 Asn Ala Thr Tyr Lys 200 Glu Ile Gly Leu 205 Thr Cys Glu
 Ala Thr Val Asn Gly His Leu 215 Tyr Lys Thr Asn Tyr 220 Leu Thr His Arg
 Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser 235 Thr Pro Arg Pro Val
 225 Lys Leu Leu Arg Gly 245 His Thr Leu Val Leu 250 Asn Cys Thr Ala Thr 240 Thr
 Pro Leu Asn Thr 260 Arg Val Gln Met Thr 265 Trp Ser Tyr Pro Asp Glu Lys
 Asn Lys Arg Ala Ser Val Arg Arg 280 Ile Asp Gln Ser 285 Asn Ser His
 Ala Asn 275 Ile Phe Tyr Ser Val 295 Leu Thr Ile Asp Lys 300 Met Gln Asn Lys
 Asp 290 Lys Gly Leu Tyr Thr 310 Cys Arg Val Arg Ser 315 Gly Pro Ser Phe Lys
 305 Ser Val Asn Thr 325 Val His Ile Tyr Asp 330 Lys Ala Phe Ile Thr 320 Val
 Lys His Arg Lys 340 Gln Gln Val Leu Glu 345 Thr Val Ala Gly Lys Arg Ser
 Tyr Arg Leu Ser Met Lys Val Lys 360 Ala Phe Pro Ser Pro 365 Glu Val Val
 Trp Leu Lys Asp Gly Leu Pro 375 Ala Thr Glu Lys Ser 380 Ala Arg Tyr Leu
 Thr 370 Arg Gly Tyr Ser Leu 390 Ile Ile Lys Asp Val Thr Glu Glu Asp Ala
 385 Gly Asn Tyr Thr 405 Leu Leu Ser Ile Lys 395 Gln Ser Asn Val Phe 400 Lys
 Asn Leu Thr Ala 420 Thr Leu Ile Val Asn 425 Val Lys Pro Gln Ile 415 Tyr Glu
 Lys Ala Val Ser Ser Phe Pro Asp 440 Pro Ala Leu Tyr Pro 445 Leu Gly Ser
 Arg Gln Ile Leu Thr Cys Thr Ala Tyr Gly Ile Pro 460 Gln Pro Thr Ile
 450 Lys Trp Phe Trp His Pro 470 Cys Asn His Asn His Ser Glu Ala Arg Cys
 465 Asp Phe Cys Ser Asn 485 Asn Glu Glu Ser Phe 475 Ile Leu Asp Ala Asp 480 Ser
 490

FIG. 3A

RECTIFIED SHEET (RULE 91)

4/8

Asn	Met	Gly	Asn ⁵⁰⁰	Arg	Ile	Glu	Ser	Ile ⁵⁰⁵	Thr	Gln	Arg	Met	Ala ⁵¹⁰	Ile	Ile
Glu	Gly	Lys ⁵¹⁵	Asn	Lys	Met	Ala	Ser ⁵²⁰	Thr	Leu	Val	Val	Ala ⁵²⁵	Asp	Ser	Arg
Ile	Ser ⁵³⁰	Gly	Ile	Tyr	Ile	Cys ⁵³⁵	Ile	Ala	Ser	Asn	Lys ⁵⁴⁰	Val	Gly	Thr	Val
Gly ⁵⁴⁵	Arg	Asn	Ile	Ser	Phe ⁵⁵⁰	Tyr	Ile	Thr	Asp	Val ⁵⁵⁵	Pro	Asn	Gly	Phe	His ⁵⁶⁰
Val	Asn	Leu	Glu	Lys ⁵⁶⁵	Met	Pro	Thr	Glu	Gly ⁵⁷⁰	Glu	Asp	Leu	Lys	Leu ⁵⁷⁵	Ser
Cys	Thr	Val	Asn ⁵⁸⁰	Lys	Phe	Leu	Tyr	Arg ⁵⁸⁵	Asp	Val	Thr	Trp	Ile ⁵⁹⁰	Leu	Leu
Arg	Thr	Val ⁵⁹⁵	Asn	Asn	Arg	Thr	Met ⁶⁰⁰	His	Tyr	Ser	Ile	Ser ⁶⁰⁵	Lys	Gln	Lys
Met	Ala ⁶¹⁰	Ile	Thr	Lys	Glu	His ⁶¹⁵	Ser	Ile	Thr	Leu	Asn ⁶²⁰	Leu	Thr	Ile	Met
Asn ⁶²⁵	Val	Ser	Leu	Gln	Asp ⁶³⁰	Ser	Gly	Thr	Tyr	Ala ⁶³⁵	Cys	Arg	Ala	Arg	Asn ⁶⁴⁰
Val	Tyr	Thr	Gly	Glu ⁶⁴⁵	Glu	Ile	Leu	Gln	Lys ⁶⁵⁰	Lys	Glu	Ile	Thr	Ile ⁶⁵⁵	Arg
Gly	Glu	His	Cys ⁶⁶⁰	Asn	Lys	Lys	Ala	Val ⁶⁶⁵	Phe	Ser	Arg	Ile	Ser ⁶⁷⁰	Lys	Phe
Lys	Ser	Thr ⁶⁷⁵	Arg	Asn	Asp	Cys	Thr ⁶⁸⁰	Thr	Gln	Ser	Asn	Val ⁶⁸⁵	Lys	His	

FIG. 3B

5/8

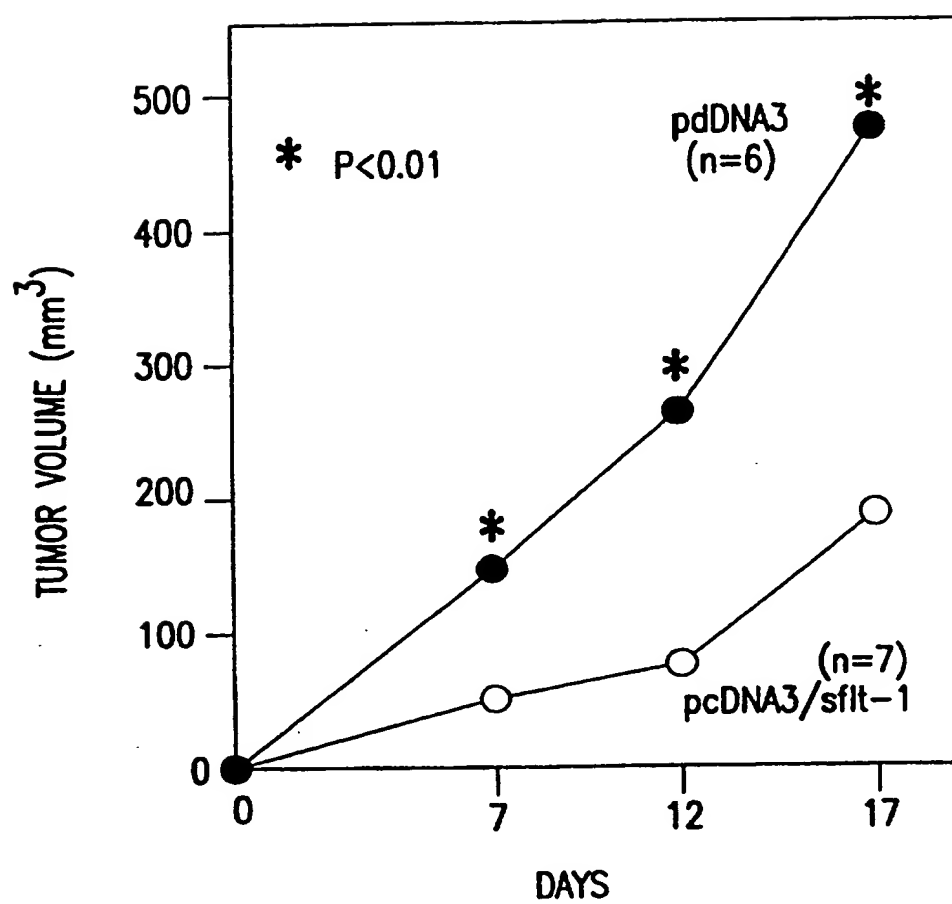


FIG.4

6/8

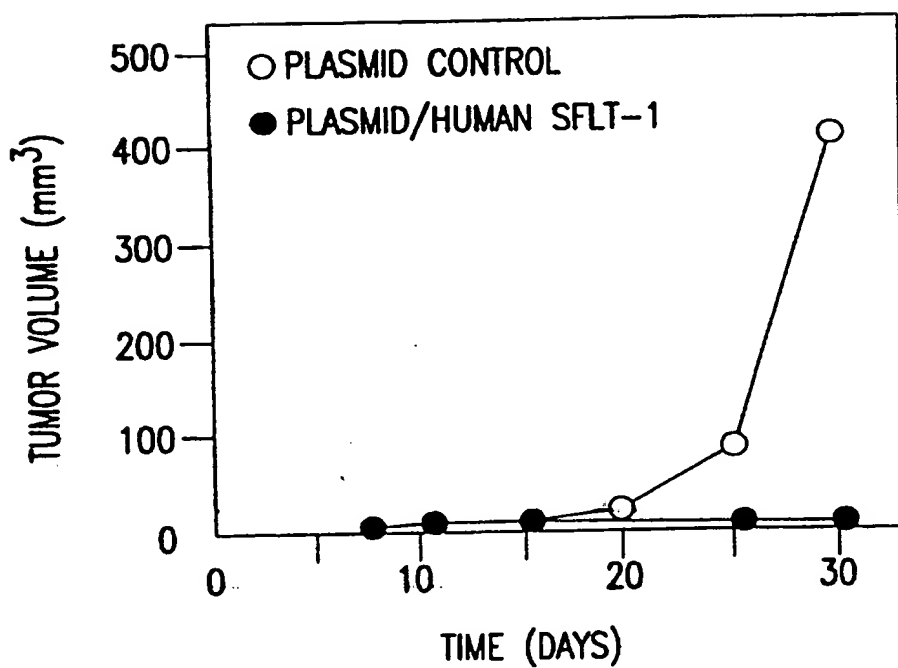


FIG.5

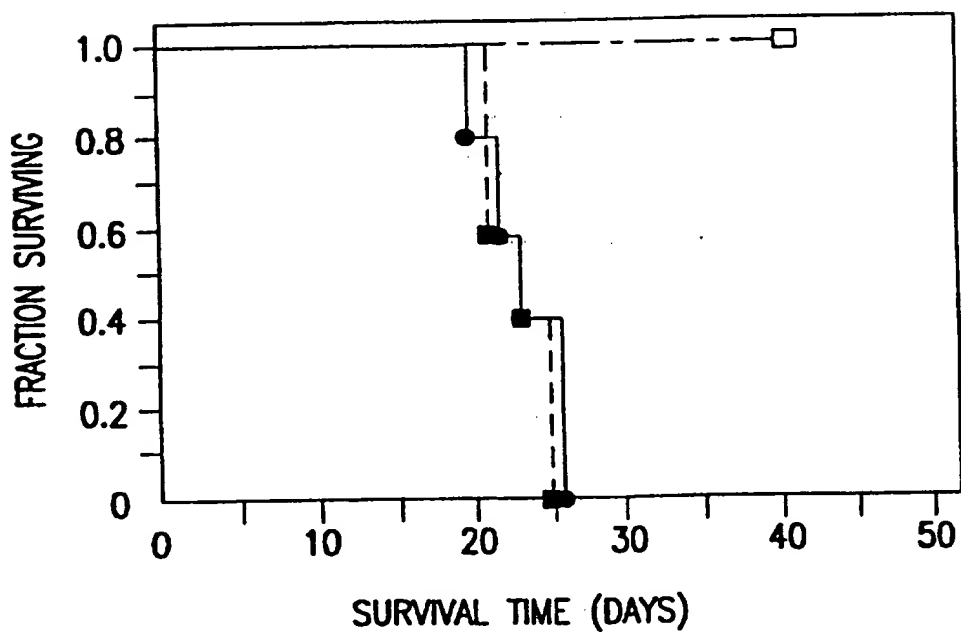
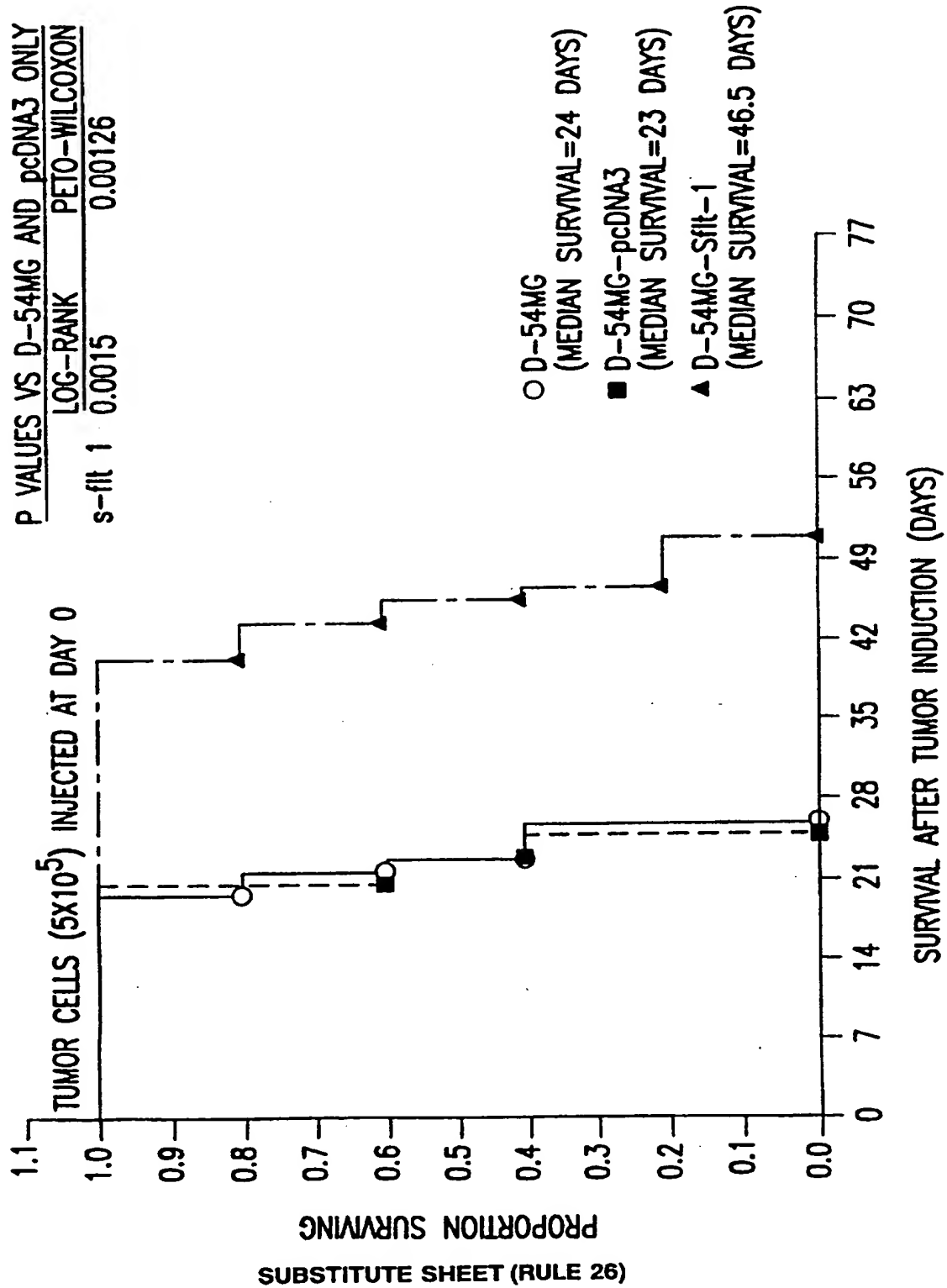


FIG.6

SUBSTITUTE SHEET (RULE 28)

7/8



8/8

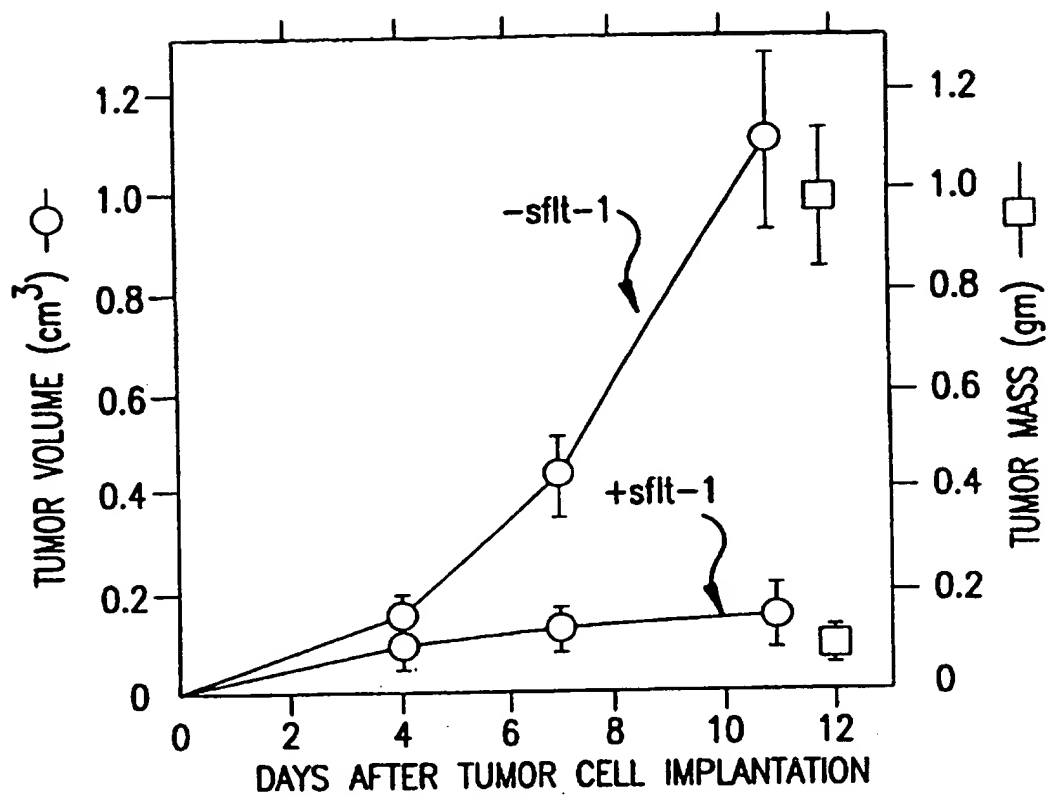


FIG.8

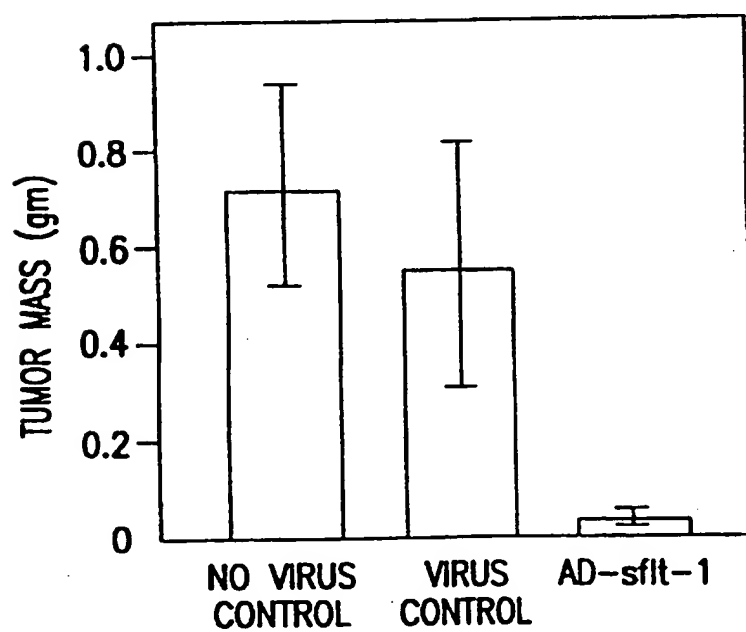


FIG.9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17044**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 48/00, 49/00; C12Q 1/68, 1/70; C12N 15/85, 15/86

US CL :424/93.2, 93.6; 435/5, 6, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2, 93.6; 435/5, 6, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Medline, Biotech

Search terms: gene therapy, adenovirus, angiogenesis, tyrosine kinase receptor, VEGF, FLT-1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,194,596 A (TISCHER et al.) 16 March 1993, columns 1-2.	1-6, 19-23
A	MILLAUER et al. Glioblastoma Growth Inhibited in vivo by a Dominant-Negative Flk-1 Mutant. Nature. 10 February 1994, Vol. 367, pages 576-579, especially pages 577-578.	1-25
A	AIELLO et al. Suppression of Retinal Neovascularization in vivo by Inhibition of Vascular Endothelial Growth Factor (VEGF) Using Soluble VEGF-Receptor Chimeric Proteins. Proceedings of the National Academy of Science, USA. November 1995, Vol. 92, pages 10457-10461, especially page 10460.	1-18

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 NOVEMBER 1997

Date of mailing of the international search report

23 DEC 1997

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INTERNATIONAL SEARCH REPORT**International application No.**
PCT/US97/17044

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KENDALL et al. Inhibition of Vascular Endothelial Cell Growth Factor Activity by an Endogenously Encoded Soluble Receptor. Proceedings of the National Academy of Science, USA. Vol. 90, pages 10705-10709, especially 10708-10709.	1-25

